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Research Paper

Inhalable Antibiotic Delivery Using a Dry Powder Co-delivering Recombinant Deoxyribonuclease and Ciprofloxacin for Treatment of Cystic Fibrosis

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Purpose. To achieve efficient antibiotic delivery to the cystic fibrosis (CF) airway using a single inhalable powder co-encapsulating a mucolytic and an antibiotic.
Methods. Inhalable dry powders containing deoxyribonuclease and/or ciprofloxacin (DNase, Cipro, and DNase/Cipro powders) were produced by spray-drying with dipalmitylphosphatidylcholine, albumin, and lactose as excipients, and their antibacterial effects were evaluated using the artificial sputum model.
Results. All powders showed mass median aerodynamic diameters below 5 μm. Both drugs were loaded in the dry powders without loss in quantity and activity. Dry powders containing DNase significantly decreased the storage modulus of the artificial sputum medium in less than 30 min. When applied to artificial sputum laden with *Pseudomonas aeruginosa*, Cipro/DNase powder showed better antibacterial activity than Cipro powder. The higher activity of the Cipro/DNase powder is attributable to the mucolytic activity of DNase, which promotes penetration of the dry powder into the artificial sputum and efficient dissolution and diffusion of ciprofloxacin.
Conclusions. Inhalational delivery of antibiotics to the CF airway can be optimized when the sputum barrier is concomitantly addressed. Co-delivery of antibiotics and DNase using an inhalable particle system may be a promising strategy for local antipseudomonal therapy in the CF airway.

KEY WORDS: artificial sputum; co-delivery; cystic fibrosis; DNase; inhalable dry powder.

INTRODUCTION

Cystic fibrosis (CF) is a common genetic disorder, affecting 1/3,200 Caucasian-American (1), 1/15,000 African-American, and 1/32,000 Asian-American newborns (2). Steady gains in the understanding of the CF pathophysiology have improved its long-term outcomes. However, CF continues to be a crippling disease that significantly affects patients' quality and duration of life.

Fundamentally a wide-spread abnormality of transepithelial chloride transport and fluid secretion, CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which encodes a chloride channel located in the apical membrane of epithelial cells (3). CFTR malfunction results in dehydrated, viscous mucus (4,5), which affects several organ systems, including the lung, the pancreas, and the gastrointestinal and reproductive tracts (6). Severe lung disease is the main cause of morbidity and mortality in CF patients (3). High mucus viscoelasticity results in ineffective cough and poor airway clearance, leading to progressive airway obstruction by thick sputum. This sets the stage for chronic bacterial infection and inflammation, begetting more sputum, and resulting in a vicious cycle of chronic infection, inflammation, and airway damage, which leads to progressive lung destruction and respiratory failure (3).

Gene or protein replacement aimed to restore the CFTR activity in the involved tissues and organs would be the ideal treatment of CF; however, it is unavailable. Current CF therapy is focused on attenuating disease progression and delaying the onset of irreversible lung damage by controlling airway infection/inflammation and improving mucus clearance (4). Some of the therapeutic agents that form the mainstay of CF therapy, notably the mucolytic agent Dornase alpha (recombinant human deoxyribonuclease) and several antibiotics, are delivered directly to the airway as aerosols. The benefit of aerosol therapy is that it is simple to administer, yet it delivers the drugs directly to the desired site of action, diminishing side effects and decreasing the need for systemic, especially parenteral, therapy. Inhaled antibiotics (7,8) and Dornase alpha (9,10) have decreased the number of exacerbation and shortened hospitalizations

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among patients with CF, reduced health-care expenditures, increased patient satisfaction, and improved physiological and clinical outcomes.

However, thick, tenacious sputum in the CF airway poses a formidable barrier to the effective inhalational delivery of antibiotics, since *Pseudomonas* and other resident pulmonary bacteria are often insulated from the inhaled antibiotics by a poorly-penetrable sputum layer (4,11,12). It is hypothesized that co-delivery of inhaled antibiotics with a mucolytic to decrease sputum viscoelasticity may improve antibiotic penetration into the sputum, increasing antibacterial potency and reducing the requisite dose. Here, an inhalable dry powder co-delivering recombinant human deoxyribonuclease (DNase, mucolytic) and ciprofloxacin (antibiotic) is produced by spray-drying. The effect of spray-dried DNase on sputum rheology is examined using the artificial sputum medium model. The antibacterial activity of the dry powder containing DNase and ciprofloxacin is compared with that of powders containing each component alone using the *Pseudomonas*-laden artificial sputum.

MATERIALS AND METHODS

Materials

Ciprofloxacin, tryptic soy broth, tryptic soy agar, cellulase from *Aspergillus niger* and amino acids (1 g of each: l-alanine, l-arginine hydrochloride, l-asparagine, l-aspartic acid, l-cysteine, l-cystine, l-glutamic acid, l-glutamine, glycine, l-histidine hydrochloride, l-4-hydroxyproline, l-isoleucine, l-leucine, l-lysine hydrochloride, l-methionine, l-phenylalanine, l-proline, l-serine, l-threonine, l-tryptophan, l-tyrosine, l-valine) were purchased from Fluka (St. Louis, MO). Bovine serum albumin, porcine stomach mucin (Type II), methyl green, thimerosal, Tween® 20, sodium dodecyl sulfate (SDS), phosphate buffered saline (PBS), diethylenetriaminepentaacetic acid (DTPA) and deoxyribonucleic acid sodium salt from salmon (DNA) were purchased from Sigma (St. Louis, MO). Dipalmitoylphosphatidylcholine (DPPC) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Dornase alfa (Pulmozyme®, Genentech, Inc) was purchased through Pharmacy Department at Advocate Lutheran General Children's Hospital (Park Ridge, IL). Chloramphenicol was purchased from Alfa Aesar (Ward Hill, MA). Egg yolk enrichment (50%) 100 ml was purchased from Remel (Lenexa, KS), and 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) from J.T.Baker. All other chemicals were purchased from Mallinckrodt Baker, Inc. (Philipsburg, NJ). *Pseudomonas aeruginosa* (*P. aeruginosa*) (ATCC 27853) was purchased from the American Type Culture Collection (Manassas, VA). Mueller-Hinton broth was purchased from BD Difco (Sparks, MD).

Preparation of Spray-Dried Powders

Composition of spray-dried powders prepared for this study is summarized in Table I. In all cases, the inactive excipients consisted of DPPC, albumin, and lactose in the ratio of 3:1:1 by weight, and combinations of drug(s) and inactive ingredients were prepared as 2 mg/mL solutions in 70% ethanol. For the Blank powder, the feed solution was prepared by mixing DPPC dissolved in 95% ethanol and albumin and lactose dissolved in water. The feed solution was then spray-dried using the LabPlant SD-05 Spray Dryer (LabPlant Ltd., UK) equipped with a 1-mm orifice nozzle. The solution was fed at the rate of 9 mL/min, and the inlet temperature was 90°C (outlet temperature: 50–55°C).

The Cipro/DNase powder was prepared using a feed solution containing both DNase solution and ciprofloxacin. Here, ciprofloxacin was first added to a small amount of water and homogenized with a Sonics Vibra-Cell Ultrasonic Processor (Sonics & Materials Inc., Newtown, CT) for 8 min alternating on and off (4 min total sonication time) at 50% amplitude and then combined with the aqueous solution of albumin and lactose. This aqueous ciprofloxacin suspension was then added to the ethanol solution of DPPC. DNase solution (Dornase alfa, 1.0 mg/mL) 2.32 mL was added to the feed solution shortly before the spray drying. To prevent sedimentation of ciprofloxacin particles, the feed solution was stirred on a magnetic stirrer throughout the spray-drying process.

To compare with the Cipro/DNase powder, each drug was spray-dried to produce DNase or Cipro powder. To prepare the DNase powder, 2.32 mL of DNase solution was added to the solution of the inactive excipients prior to spray drying. For the Cipro powder, ciprofloxacin was homogenized in water using the ultrasonic processor, combined with the aqueous solution of albumin and lactose, and then added to the ethanol solution of DPPC. The feed solution was stirred throughout the spray-drying process.

Characterization of Spray-Dried Powders

Scanning Electron Microscopy

The morphology of the prepared dry powders was examined using scanning electron microscopy. Dry powders were attached to specimen stubs using double-sided tape and sputter-coated with gold-palladium in the presence of argon gas using a Hummer I sputter coater (Anatech Ltd.). Dry

Table I. Summary of Spray-Dried Powders

	Target drug content		Actual drug content				
Powder	Cipro-floxacin (μg/mg powder)	DNase (μg/mg powder)	Cipro-floxacin (μg/mg powder)	DNase (μg/mg powder)	DNase (% activity)	FPF (%)	MMAD (μm)
Blank	–	–	–	–	–	34.6±6.1	4.1±0.5
Cipro	50	–	45.6±1.8	–	–	53.1±5.6	3.0±0.3
DNase	–	2.9	–	3.2±0.2	111.1±8.5	25.0±7.3	4.6±0.2
Cipro/DNase	50	2.9	50.2±4.0	3.0±0.1	102.9±3.2	49.5±2.2	3.1±0.3

powders were imaged with a JEOL JSM-840 scanning electron microscope (JEOL USA, Inc.) using a 5 kV accelerating voltage, a 10 mm working distance, a 70 μm objective aperture, and a probe current of 3×10^{-11} amps.

Anderson Cascade Impactor

Aerodynamic particle size distribution was determined using the eight-stage Mark II Anderson Cascade Impactor (ACI). Powder samples (10 mg) were manually loaded into hard gelatin capsules (size 3), which were put in a Rotahaler and split open to release the particles. Glass fiber filters were placed on the ACI stages to prevent particle bounce or re-entrainment (13). Each set of powder was drawn through the induction port into the ACI operated at a flow rate of 28.3 L/min for 10 s, which allowed the aspiration of approximately 4 L of air into the apparatus. The amount of particles deposited at each impaction stage was determined by measuring the difference in weight of glass fiber filters (for the filter stage, pore size <1 μm, ThermoFisher; for all other stages, pore size 1 μm, Pall Corp.). The effective cutoff aerodynamic diameters for each stage were Stage 0, 9 μm; Stage 1, 5.8 μm; Stage 2, 4.7 μm; Stage 3, 3.3 μm; Stage 4, 2.1 μm; Stage 5, 1.1 μm; Stage 6, 0.65 μm; Stage 7, 0.43 μm. The fine particle fraction (FPF) was defined as the amount of powder with an aerodynamic size <4.7 μm (particles deposited at stage 3 and lower) divided by the initial total powder loaded in the Rotahaler (10 mg, nominal dose). The cumulative mass of powder less than effective cutoff diameter as percent of total mass recovered in the ACI was plotted against the effective cutoff diameter. The mass median aerodynamic diameter (MMAD) was defined on this graph as the particle size at which the line crossed the 50th percentile.

Determination of the Drug Content in Dry Powder

For determination of ciprofloxacin content in dry powder, 1.5 mg of Cipro or Cipro/DNase powder was dissolved in 1 mL of HCl solution (pH 2) containing 0.5% SDS. This mixture was agitated using a vortex mixer to dissolve the powder and centrifuged at 8000 rpm for 5 min. The supernatant was then diluted 5 times with water. The concentration of ciprofloxacin in the supernatant was determined using High Pressure Liquid Chromatography (HPLC 1100 series, Agilent Technologies, Palo Alto, CA) and an Atlantis analytical column (dC18; 4.6×250 mm; particle size 5 μm). The mobile phase was a mixture of 10 mM phosphate buffer (pH 2.1) and acetonitrile (with an increasing ratio of acetonitrile from 20% to 70% over 8 min). The flow rate was 1 mL/min. Five μL of each sample were injected into the pre-equilibrated column followed by 10 min of wash with the mobile phase. The UV detector was set at 275 nm. Retention time of ciprofloxacin was 4.3 min.

For determination of DNase content in dry powder, 1.5 mg of DNase or Cipro/DNase powder was dissolved in 1 mL PBS (pH 7.4) and gently mixed. After 1 h of incubation at 37°C, the mixture was centrifuged at 8000 rpm for 5 min. Fifty microliters of supernatant were analyzed by HPLC and a Vydac 214TP54 column (C4, 300 Å, 4.6×250 mm; particle size 5 μm). The mobile phase consisted of solvent A (acetonitrile containing 0.1% trifluoroacetic acid) and solvent B (water containing 0.1% trifluoroacetic acid) delivered at 1 mL/min in a linear gradient: 0–40 min, 5% A to 50% A; 40–45 min, 50% A; 45–47 min, 50% A to 5% A; 47–62 min, 5% A. The UV detector was set at 214 nm. Retention time of DNase was 40.9 min.

To confirm the consistent loading of the two drugs in the Cipro/DNase powder, the powder was run through the ACI, and the drug contents in the powder collected at each ACI stage were determined. Two capsules of 10 mg powder were discharged into the ACI (total 20 mg per test) without using glass fiber filters on the stages. Particles on each stage were collected and accurately weighed, dispersed in 1 mL PBS, and incubated overnight. The drug contents in the powder suspension were determined by measuring the ciprofloxacin concentration and the DNase activity (described below). The measurements were performed in triplicate.

Drug Release from Dry Powders

To evaluate the bioactivity of the spray-dried drug, the dry powder was incubated in PBS to release the drug. To ensure that the ciprofloxacin release was not limited by its solubility in the medium (~200 μg/mL (14)), 1 mL PBS was used to disperse 1.5 mg powder (equivalent to 75 μg of ciprofloxacin), and the release medium was replaced with fresh PBS frequently. Thus, the ciprofloxacin concentration in PBS was always maintained below its solubility in PBS at 37°C. *First,* 1.5 mg of dry powder was accurately weighed, suspended in 1 mL of PBS, and incubated in 37°C with constant agitation. After 0.5 h of incubation, the powder suspension was centrifuged at 8000 rpm for 5 min to separate 0.8 mL supernatant, and an equal volume of fresh PBS was replaced. This sampling procedure was repeated at 1, 2, 3, and 5 h after incubation. The sampled supernatants were stored at −20°C and analyzed with HPLC (DNase, ciprofloxacin), colorimetric activity assay (DNase), or broth microdilution method (ciprofloxacin), to determine the concentration and bioactivity of each drug.

This method was used to maximize the drug recovery from the powders without bringing the drug concentration below the detection limit. It may not predict the drug release kinetics in the respiratory tract, where the inhaled powder would be placed at the interface of air and mucus-covered moist lung tissues rather than in liquid.

Determination of DNase Activity

The activity of DNase in the dry powder was measured using a colorimetric activity assay described in the literature, which can sensitively determine the activity of the enzyme in the range of 0.1–10 μg/mL (15,16). Here, a complex of DNA and methyl green is incubated with DNase. DNA hydrolysis by DNase releases free methyl green from the DNA-methyl green complex and decreases the absorbance at 620 nm (A_{620}). The rate of absorbance decrease (ΔA_{620}/time) is proportional to the DNase activity.

A DNA stock solution (2 mg/mL) was prepared in buffer A (25 mmol/L HEPES, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), pH 7.5) and gently mixed until DNA was completely dissolved. A 0.4% methyl green solution in buffer B (20 mmol/L acetate-NaOH, pH 4.2) was repeatedly extracted with chloroform until the organic layer was color-

less to ensure complete removal of trace crystal violet. A DNA-methyl green substrate solution was then prepared by mixing 10 mL of DNA stock solution, 600 μL of 0.4% methyl green solution, 2.75 mL of buffer C (25 mmol/L HEPES, 4 mmol/L $CaCl_2$, 4 mmol/L $MgCl_2$, 0.1% bovine serum albumin, 0.01% Thimerosal, 0.05% Tween 20, pH 7.5), and 10 μL of 30% hydrogen peroxide. The substrate solution was equilibrated overnight at room temperature. To measure DNase activity, 90 μL of the sample solution or standard solutions with known DNase activity, 90 μL of the DNA-methyl green substrate solution, and 100 μL of buffer C were mixed in a 96-well plate, and change in A_{620} was immediately measured at 37°C over 200 consecutive time intervals of 40 s using a Tecan Spectrafluor Plus microplate reader (Männedorf, Switzerland). The calibration curve was generated by correlating the ΔA_{620}/min with the concentrations of the DNase standard solutions. Since the enzyme activity varied significantly with the solvent system, DNase standards were prepared in PBS for parallel comparison with sample solutions.

Determination of Minimal Inhibitory Concentration of Spray-Dried Ciprofloxacin

The minimal inhibitory concentration (MIC) of ciprofloxacin in dry powder was determined in accordance with the Clinical and Laboratory Standards Institute (CLSI) guideline for broth microdilution procedures (17). Briefly, *P. aeruginosa* (ATCC 27853) was grown in Mueller-Hinton broth for 12 h. The bacterial suspension was diluted in Mueller-Hinton broth to yield an inoculum concentration of 5×10^6 colony forming unit (CFU) per mL. The absorbance at 420 nm was 0.9 for 5×10^6 CFU/mL. Ten microliters of the inoculum were added to wells of a 96-well plate, which contained 100 μL of serially-diluted solutions of spray-dried ciprofloxacin or standard ciprofloxacin (concentration range: 0.0086 to 8.8 μg/mL) to make the final concentration ranging from 0.008 to 8 μg/mL. Bacterial growth was determined after overnight incubation at 37°C. The MIC was defined as the lowest drug dilution with no visible growth, as indicated by the lack of absorbance at 430 nm. The MIC measurement was performed in triplicate.

Preparation of Artificial Sputum Medium

Artificial sputum medium (ASM) was prepared as previously described (18) with modifications. Fifty milliliters of sterile ASM were prepared by dissolving 500 mg of DNA (sterilized with overnight exposure to a germicidal lamp in a laminar flow hood) in 32.5 mL DNase-free water, complemented with 250 μL of sterile egg yolk emulsion and an autoclaved solution of 250 mg mucin, 0.295 mg DTPA, 250 mg NaCl, 110 mg KCl, 12.5 mg amino acids dissolved in 17.5 mL water. The pH of the ASM was adjusted to 7.0 using a sterile NaOH solution.

Rheological Analysis of Artificial Sputum Medium

1.6 mg of Blank, Cipro, DNase or Cipro/DNase powder was placed on 1 mL of ASM, and incubated at 37°C. After 1, 3, 7, or 11.5 h, the ASM samples were sampled and stored at −80°C until analysis. Rheological properties of the ASMs were determined as previously described (19) using the AR 2000 rheometer (TA Instruments, Leatherhead, Surrey, UK). ASMs, with or without the powder treatment, were equilibrated to 20°C and placed on the temperature-controlled Peltier plate. Forty-mm parallel plate geometry was then placed on the samples, maintaining a gap width of 250 μm. A solvent trap with water was used to prevent the dehydration of the samples. All the measurements were performed at 20°C. A stress sweep (σ, 0.1–10 Pa) was first performed on non-treated ASM at 1 Hz in a dynamic oscillatory mode to determine the linear viscoelastic region, which was found to be 0.1–4 Pa. Subsequently, a frequency sweep (0.1–10 Hz) was performed for all ASM samples at a constant stress of 0.15 Pa (σ) to determine their storage moduli (G').

Preparation of Artificial Sputum

A single colony of *P. aeruginosa* grown on a tryptic soy agar plate was isolated and seeded in 25 mL of tryptic soy broth followed by incubation at 37°C with gentle shaking. When the optical density of the broth at 420 nm reached 0.34, 10 μL of the broth was added to 1 mL of sterile ASM and incubated at 37°C for 2 h prior to the treatment with dry powders. The *P. aeruginosa*-laden ASM is referred to as the artificial sputum.

Antibacterial Activity of Dry Powders in Artificial Sputum

To compare antipseudomonal efficacy of all the dry powders, 1.6 mg of each powder (Blank, Cipro, DNase, or Cipro/DNase) was placed on 1 mL of the artificial sputum and incubated at 37°C for 1 or 2 h, after which the powder-treated sputum was exposed to 1 mL of dilution medium (2 mg/mL cellulase, 400 μg/mL chloramphenicol in 0.05 mol/L citrate buffer, pH 4.6) for 30 min at 37°C (18). Ten μL of the diluted artificial sputum was further diluted with tryptic soy broth as needed, plated on a tryptic soy agar plate, and incubated at 37°C overnight, after which the bacterial colonies were counted. To generate the dose-response curve of the Cipro and Cipro/DNase powders against *P. aeruginosa* in the artificial sputum, 0.16, 0.5, 1.6, 5, or 16 mg of each powder (corresponding to 8, 25, 80, 250, or 800 μg of ciprofloxacin) were respectively placed on the artificial sputum, which was then treated as described above.

Statistical Analysis

All data were expressed as averages with standard deviations. ANOVA was used to determine statistical difference among the groups, and then pair-wise comparison was made using the Student t-test. A p-value of <0.05 on a 2-tailed test was considered statistically significant.

RESULTS

Preparation and Characterization of Spray-Dried Powders

Preparation of Spray-Dried Powders

Since the stability of DNase can be negatively influenced by high temperature (20) and extreme pH (especially when it

is coupled with a thermal effect) (20,21), the inlet temperature was maintained relatively low (90°C) and pH of the feed solution was neutral. As solubility of ciprofloxacin in the ethanolic solution of excipients with a neutral pH was lower than its desired concentration, the undissolved portion of ciprofloxacin was homogenized using a probe sonicator. After a 4-minute sonication, the particle size of ciprofloxacin was 324.9±101.0 nm. The feed solution was stirred on a magnetic stirrer throughout the spray drying process to prevent sedimentation of ciprofloxacin particles. To evaluate the homogeneity of the ciprofloxacin distribution in the Cipro/DNase powder, the spray-dried powder was sampled from various locations of the collection bottle and analyzed for the ciprofloxacin content. The powder samples collected from the neck, wall, and bottom of the collection bottle contained 104.4±9.9%, 97.3±8.6%, and 110.1±6.5% of the expected ciprofloxacin, respectively (averages ± standard deviations of 3 independent batches; $p>0.05$ by ANOVA). This result confirms that the ciprofloxacin particles were not separated during the drying process and were homogeneously distributed in the spray-dried powder.

Particle Morphology

The dry powders showed similar appearance irrespective of the loaded drugs, as shown in Fig. 1, indicating that the particle morphology was dictated by the inactive ingredients (DPPC, lactose, and albumin) and not influenced by the presence of drugs. This result was anticipated, as the total drug content in this study was less than 5.5% *w/w* of the particles, and even particles with ~50% drug content were previously reported to have similar morphology as blank particles (22). They consisted of small toroidal particles (1–3 μm in diameter) and larger spherical ones (~10 μm), which were hollow, as shown in the inset of Fig. 1A. There was no significant difference among the powders in roughness of the particle surface (Fig. 1B).

Aerodynamic Properties

Aerodynamic properties of Blank, Cipro, DNase or Cipro/DNase powders were evaluated using the Anderson Cascade Impactor (Table I). All the powders exhibited MMADs below 5 μm, which is desirable for inhalation (23–25). FPF was relatively high when ciprofloxacin was included in the powder. While DNase powder had similar FPF to Blank powder ($p>0.05$), Cipro powder showed a significantly higher FPF than Blank powder ($p<0.05$), and Cipro/DNase powder showed a significantly higher FPF than those of Blank powder ($p<0.05$) and DNase powder ($p<0.01$).

Drug Content in Dry Powders

Table I summarizes the drug content in Cipro, DNase, and Cipro/DNase powders. HPLC analysis shows that both ciprofloxacin and DNase were loaded in the dry powder without loss, respectively as well as collectively. Upon incubation in PBS at 37°C, both ciprofloxacin and DNase were completely recovered from the powders in less than 1 h (Supplementary Fig. 1).

Both ciprofloxacin and DNase were consistently loaded in the Cipro/DNase powder (Supplementary Fig. 2). The Cipro/DNase powder collected on each stage of the ACI contained 85.4–94.9% of the expected ciprofloxacin and 88.7–106.6% of the expected DNase. There was no significant difference among the stages (for both drugs, $p>0.05$ by ANOVA). This result indicates that the Cipro/DNase powder would deliver consistent amounts of ciprofloxacin and DNase to all levels of the airways.

Activity of DNase and Ciprofloxacin Recovered from Dry Powders

Activity of the spray-dried DNase was tested by monitoring DNA-methyl green degradation. DNase recovered from DNase and Cipro/DNase powders maintained 111.1±8.5% and 102.9±3.2% of the expected activity, respectively, indicating that it was unaffected by the spray-drying process and by the presence of ciprofloxacin and excipients. Activity of the spray-dried ciprofloxacin was tested by measuring and comparing its MIC for *P. aeruginosa* (ATCC 27853) to that of standard ciprofloxacin. Both ciprofloxacin recovered from Cipro or Cipro/DNase powder and the standard ciprofloxacin showed the MIC of 0.25 μg/mL (Supplementary Fig. 3),

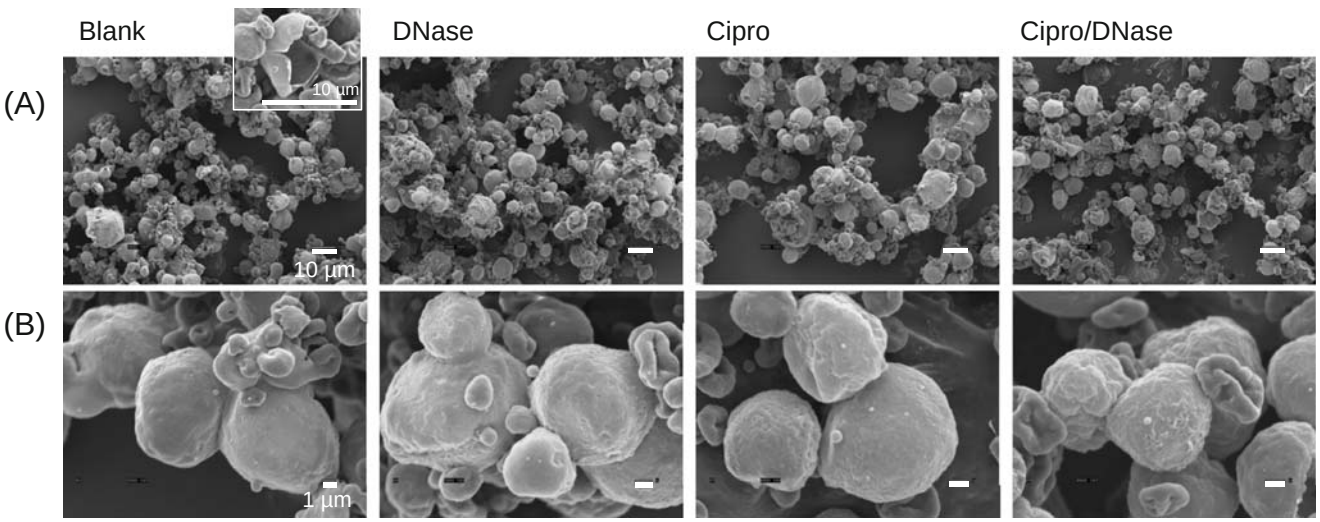

Fig. 1. Scanning electron micrographs of spray-dried powders. (**A**) Low magnification. Scale bar = 10 μm; (**B**) High magnification. Scale bar = 1 μm.

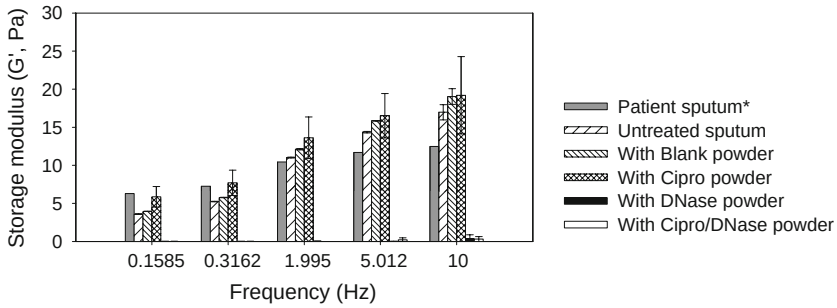

Fig. 2. Effect of spray-dried powders on storage modulus (G') of artificial sputum after 1 h incubation. Patient sputum* data were taken from the reference (19). Except for the patient sputum*, data are expressed as averages with standard deviations of 3 independent measurements.

consistent with the reported value (26). These results confirm that the bioactivity of each drug was not compromised by the presence of the other drug and the excipients: i.e., two drugs were compatible with each other and with the inactive excipients.

Effect of Dry Powders on Rheology of Artificial Sputum Medium

In order to investigate the effect of dry powders on sputum rheology, ASM was prepared as previously described (18,27); specifically, mucin, DNA, surfactant, salts, and amino acids were added for ASM to resemble biochemical properties of CF sputum and support the growth of *P. aeruginosa* (18,27). Here, mucin and DNA serve as biopolymers that constitute the sputum network (28). Amino acids were included as they are present in elevated levels in the lungs with severe diseases (29) and known to be responsible for CF-specific phenotypes of *P. aeruginosa* biofilm (18). However, when so prepared, ASM exhibited a lower storage modulus, G', than the median value demonstrated in clinical CF sputum samples (19). Since DNA concentration is mainly responsible for the high viscoelasticity of CF sputum (30), the ASM composition was modified to include a higher concentration of DNA (10 mg/mL). With this modification, G' of the ASM approached that of CF sputum (Fig. 2). The ASM stored at 37°C maintained consistent G' up to 7 h (data not shown), subsequently becoming thinner probably due to the DNA instability. Therefore, all experiments were performed within 7 h of ASM preparation.

The effect of Blank, Cipro, DNase or Cipro/DNase powders on G' of ASM was tested over a range of frequencies (Fig. 2). Blank and Cipro powders had no significant effect on the ASM's G'. The non-DNase-containing powders placed atop the ASM did not penetrate into the underlying ASM. In contrast, after exposure to DNase or Cipro/DNase powders, the thick ASM became thin liquid in less than 30 min, and the powders were able to penetrate the ASM and dissolve in it. This confirms that DNase loaded in the spray-dried powders maintained its activity and corroborates the results of the colorimetric activity assay.

Antibacterial Activity of Dry Powders

In order to evaluate the antimicrobial activity of the powders in a clinically-relevant model, artificial sputum was created by loading the ASM with *P. aeruginosa,* the major microorganism in the CF airway beyond early childhood (31). First, antimicrobial activities of all four powders were compared at a single powder dose. Here, 1.6 mg of powder was used per 1 mL of the artificial sputum, because this amount of Cipro or Cipro/DNase powders, when fully dissolved in liquid, would generate an 80 μg/mL solution of ciprofloxacin, 320 times higher than the MIC for *P. aeruginosa* (0.25 μg/mL) (26). Previously, we mentioned that *in-vivo* antipseudomonal effect of an antibiotic was demonstrated at 32 times the MIC (22). Since the time frame in which the G' of the ASM could be

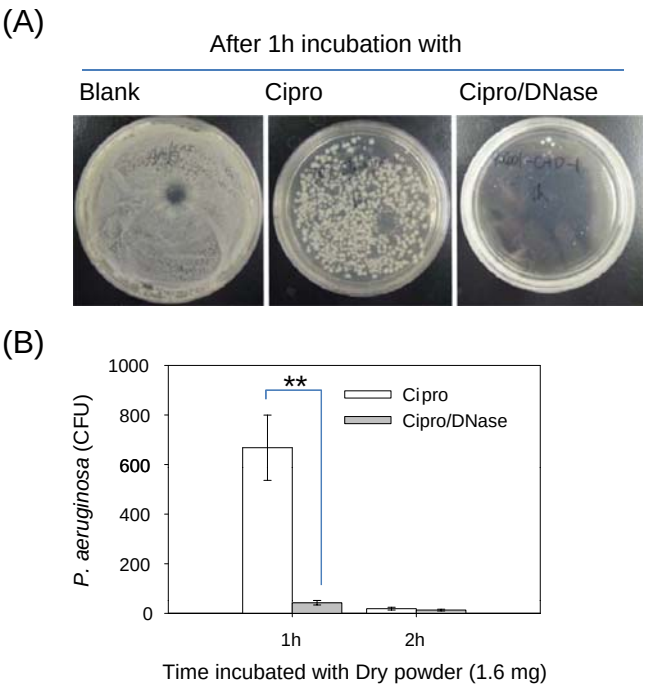

Fig. 3. Antibacterial activities of spray-dried powders. (**A**) Representative pictures of agar plates. Artificial sputum was treated with Blank, Cipro, or Cipro/DNase powders, incubated for 1 h at 37°C, diluted, and plated on the agar plates. Pictures were taken the following day. (**B**) Bacterial colony forming units (CFU) after treatment of artificial sputum with Cipro or Cipro/DNase powders. One mL of artificial sputum was treated with 1.6 mg of dry powders, incubated for 1 or 2 h at 37°C, diluted, and plated on an agar plate. Colonies were counted the next day. Data are expressed as averages with standard deviations of 3 independent batches. **: $p<0.01$ by t-test.

maintained constant was limited to 7 h, we used the amount of powder that generates a ciprofloxacin concentration far in excess, so that it compensates for the limited diffusion of ciprofloxacin in the artificial sputum and creates a concentration relevant *in vivo* in the first few hours. At 1 h after treatment, the artificial sputa treated with Blank and DNase powders showed similar numbers of bacterial colonies, and at 2 h, numbers of bacterial colonies almost doubled compared to those at 1 h, confirming that Blank and DNase powders were neither bactericidal nor bacteriostatic against *P. aeruginosa* in the artificial sputa. In contrast, the artificial sputa treated with Cipro and Cipro/DNase powders showed ~1/100 and ~1/1000 the number of bacterial colonies in the sputa treated with Blank and DNase powders, respectively (Fig. 3A). The artificial sputum treated with 1.6 mg of Cipro/DNase powder showed many fewer colonies than the artificial sputum treated with 1.6 mg of Cipro powder at 1 h, but this difference disappeared at 2 h (Fig. 3B). This suggests that the presence of DNase facilitated the initial (in <1 h) antibiotic penetration into the artificial sputum and the dissolution of the powder, and allowed the ciprofloxacin to effectively kill the bacteria. On the other hand, Cipro powder was able to kill the bacteria as well after a longer exposure (2 h). At this time, some Cipro powder was still visible on the surface of the artificial sputum, while the Cipro/DNase powder had completely disappeared 30 min after placement. Hence, the delay in bactericidal effect was likely due to initial limitations in dissolution of ciprofloxacin and its diffusion from the powder to the artificial sputum.

The difference in the antibacterial activities of Cipro and Cipro/DNase powders was also evident when dose-response curves of the two powders were compared. Both powders showed dose-dependent antibacterial activity, with the bactericidal effect of Cipro/DNase powder consistently and significantly exceeding that of Cipro powder (Fig. 4). Given that DNase did not show any antibacterial activity (Supplementary Fig. 4), this difference is attributable solely to the mucolytic activity of DNase.

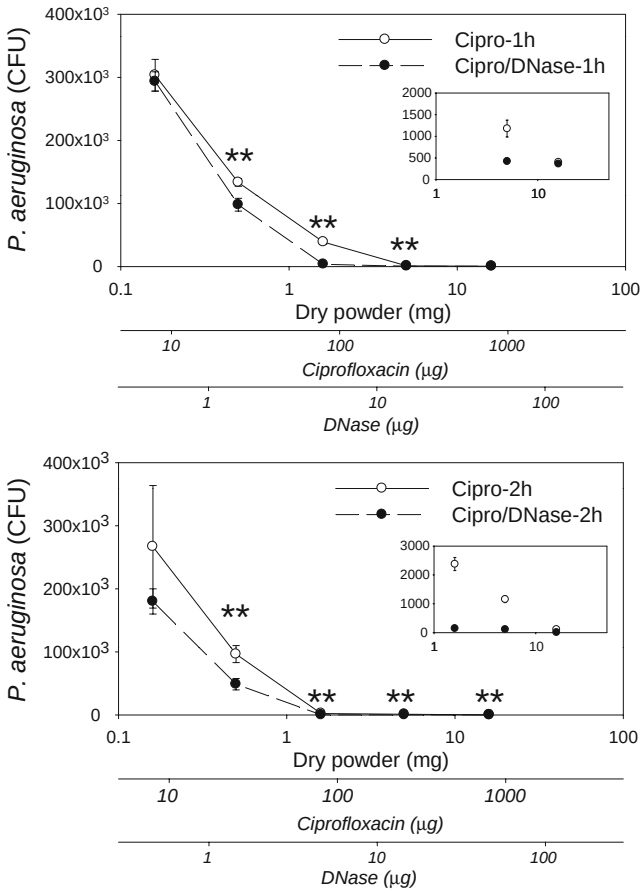

Fig. 4. Bacterial colony forming units (CFU) after treatment of artificial sputum with different amounts of Cipro or Cipro/DNase powders for 1 or 2 h at 37°C. The *first x-axis* indicates the amount of dry powder, the *second axis* indicates equivalent amount of ciprofloxacin (in Cipro or Cipro/DNase powders), and the *third axis* indicates equivalent amount of DNase (in Cipro/DNase powder). Inset graphs magnify the vertical scale to clarify the difference at higher amounts of powders (>1.6 mg). Data are expressed as averages with standard deviations of 3 independent measurements of a single batch powder. **: $p<0.01$ by t-test.

DISCUSSION

Progressive accumulation of tenacious sputum containing high concentrations of DNA and actin polymers (12,32–35) is the cornerstone of CF airway pathology. The abundant actin-DNA bundles in CF sputum support the formation of *P. aeruginosa* biofilm (12,32,36), which is resistant to the standard antibiotic treatment (12,37,38). Moreover, the presence of thick sputum and *P. aeruginosa* biofilms decreases the penetration of antibiotic aerosols to the site of action and thus limits their efficiency (4,12,39).

By co-delivering an antibiotic with DNase, which would liquefy the CF sputum, we hope to enhance penetration and dissolution/diffusion of the antibiotic. The results show that it is possible to co-deliver DNase and ciprofloxacin in a single dry-powder formulation without compromising the activity of each drug, and that such an approach enhances the antibacterial activity of ciprofloxacin in the artificial sputum model. Co-delivery of the two drugs as a single powder would ultimately potentiate the ciprofloxacin effect while reducing the dose and the time required to administer the daily regimen of antibiotics and mucolytics, leading to better microbiological outcomes, better patient compliance and, ultimately, better clinical results.

Ciprofloxacin is used as a model antibiotic due to its relatively reliable antipseudomonal activity and reported clinical utility, especially in early infection (40). Parenteral and/or enteral fluoroquinolones are routinely used in clinical practice to control chronic pseudomonal and other pulmonary infections or colonization in children (41) and adults (42) with CF. Although not FDA-approved for use in children below the age of 12, the risks of fluoroquinolones are outweighed by their benefits in this population (31). Since the inhaled route for the most part avoids the systemic side effects, it is especially attractive for children with CF, in whom fluoroquinolones may interfere with proper bone and cartilage growth and maturation, and with tendon strength (41). Several other groups, including industry, are developing inhalable fluoroquinolones as nebulizable solutions as well as dry powder formulations (43–45). The inhalable powder form of ciprofloxacin developed by Bayer Healthcare is currently in Phase I and II clinical trials for multiple indications including chronic pseudomonal lung infections

in patients with CF and those with chronic obstructive pulmonary disease (COPD) (http://www.clinicaltrial.gov/ct2/show/NCT00961038, http://www.clinicaltrial.gov/ct2/show/NCT00910351, http://www.clinicaltrial.gov/ct2/show/NCT00645788).

In delivering ciprofloxacin and DNase, a dry-powder platform consisting of DPPC, albumin, and lactose was chosen because it is well-characterized, has favorable aerodynamic properties, and is often used for inhalational delivery of a variety of drugs (13,23,46–48). Each excipient contributes to dispersibility of the resulting powder by conferring irregular shape and/or less adhesive surface. For example, DPPC confers a sponge-like shape on the particles, lactose makes smaller particles, whereas albumin makes the particles lighter (47) and less cohesive (23). Moreover, these components are either approved by the U. S. Food and Drug Administration for inhalation (lactose) or endogenous to the lung (albumin, DPPC). All dry powders had MMADs below 5 μm, which is acceptable for inhalation, and the FPFs ranged from 25% to 53% (Table I). Of note, the conditions for the ACI operation did not conform to the requirements of the United States Pharmacopeia, which requires an air flow rate higher than 28.3 L/min for DPIs with lower resistance like Rotahaler (49). Given that the lower air-flow rate yields relatively low FPFs (50,51), the FPF values reported here are likely underestimated. Despite this limitation, the relative order of their aerodynamic properties remains valid, since all powders were tested consistently. The powders containing ciprofloxacin had relatively high FPFs, consistent with the previously-reported results (22). This may be attributable to the distribution of ciprofloxacin nanoparticles in the sprayed droplets. Nanoparticles or molecules with low aqueous solubility (like ciprofloxacin) are typically unable to redistribute quickly in drying droplets (in contrast to water-soluble small molecular weight solutes) (52,53). Consequently, nanoparticles or molecules with low solubility accumulate on the surface and become a part of a shell, which deforms in various ways as the drying process continues (53). The surface accumulation of nanoparticles contained in a microparticle was visible when a large quantity (70–100% of total solid content) of nanoparticles was included (52,54,55). Similarly, the majority of ciprofloxacin is likely to deposit on the surface of dry microparticles, although it was not visible on the scanning electron micrographs (Fig. 1B), probably due to the small quantity (5%) of ciprofloxacin. The relatively heterogeneous surface should decrease cohesive interactions between microparticles and result in more dispersible powders.

While the activity of DNase is known to be negatively influenced by high temperature (20), it withstood the spray-drying process without losing the activity. The stability of spray-dried DNase may be attributed to three reasons. First, the actual heat to which the protein is exposed during the drying process is lower than the inlet temperature (90°C) due to the concurrent solvent evaporation (56). Second, the residence time of the DNase solution in the heated nozzle was 23 s at most (the residence time of the feed solution in the nozzle = nozzle internal volume (3.5±0.2 mL) / volumetric flow rate of the feed solution (9 mL/min)). Third, the excipients (e.g., lactose or albumin) may have mitigated the thermal denaturation of DNase.

The dry powder reported in this study contains 50 μg/mg (5%*w/w*) of ciprofloxacin and 2.9 μg/mg of DNase. Given that the ciprofloxacin dry powder form currently undergoing clinical trials contains 65% *w/w* ciprofloxacin in powder (http://www.clinicaltrial.gov/ct2/show/NCT00961038, http://www.clinicaltrial.gov/ct2/show/NCT00910351, http://www.clinicaltrial.gov/ct2/show/NCT00645788), the ciprofloxacin content may be considered too low for clinical applications. In this regard, it is noted that the drug contents were not limited by the excipient system or spray drying process and can be easily increased as needed (22). Nonetheless, the low ciprofloxacin content was used to determine the contribution of DNase, i.e., to distinguish the effects of Cipro powder and Cipro/DNase powder in the artificial sputum model. Dry powders with 50% *w/w* of ciprofloxacin content effectively killed the bacteria in the artificial sputum irrespective of the presence of DNase (data not shown); thus, the difference between Cipro and Cipro/DNase powder could not be determined. Using dry powders containing a low dose of ciprofloxacin, it was shown that co-delivery of DNase potentiates the antibacterial effect of ciprofloxacin, potentially reducing the dose requirement. Since no inhalable dry powder form of DNase is clinically available, the DNase content was determined based on the literature, which found that DNase was effective in decreasing the viscoelasticity of CF sputum at the final concentration of 2.9 μg/mL (19).

The Cipro/DNase powder was highly effective in decreasing the elasticity of the artificial sputum medium and preventing bacterial growth in the medium. The artificial sputum model was employed in lieu of clinical CF sputum samples because there is large interpatient variation in rheological properties of the sputum (19,35); thus, it would be difficult to compare the rheological and microbiological effects of these powders in clinical samples. The resemblance of the artificial sputum to CF sputum in biological and rheological properties was demonstrated by the literature (18) and in this study, respectively. However, it is acknowledged that the artificial sputum may not perfectly simulate the CF sputum, since many other physiologic factors are involved in clinical samples. For example, the artificial sputum does not include actin, another major component of CF sputum, which is known to form a three-dimensional network with DNA, and histones, which mediate formation of such a network (33). While DNase is a clinically effective mucolytic agent (57) and was highly effective in altering the rheology of ASM and artificial sputum in this study, it may be limited in dissociating actin-DNA networks; thus, additional components interfering with the DNA-actin polymer interactions (e.g., unfractionated heparin (19,35)) may need to be included for clinical applications. The artificial sputum model used in this study is also limited in representing the resistance of bacteria dwelling in the CF sputum to the antibiotic treatment. A sequence of developmental episodes in biofilm growth involves a number of phenotypic changes of the bacteria (58). One of the most clinically relevant changes is the increase in bacterial resistance to antibacterial agents (59,60). While our artificial sputum model is designed to mimic the rheological property of the CF sputum, it does not address the high resistance of the bacteria at the later stage of maturation. Given that *P. aeruginosa* was grown for 24 h and then grown in the ASM for 2 h before they were treated with

dry powders, they are unlikely to represent the resistant biofilm at the later maturation stage. While this study shows that overcoming the rheological barrier using co-administration of DNase contributed to potentiating ciprofloxacin, it remains to be seen whether the co-administration of ciprofloxacin and DNase would be effective in killing the highly-resistant bacteria at the later stage. For overcoming the bacterial resistance to the antibiotics, it is conceivable to co-administer multiple antibiotics (61,62) along with DNase.

CONCLUSIONS

An inhalable spray-dried powder that can efficiently deliver DNase and ciprofloxacin was produced, and its antibacterial activity in comparison with a powder containing ciprofloxacin alone was evaluated. The inclusion of DNase improved the antipseudomonal activity of the Cipro/DNase powder as compared with the Cipro powder in the artificial sputum model. The higher activity of the Cipro/DNase powder is attributable to the mucolytic activity of DNase, which promotes penetration of the dry powder into the artificial sputum and efficient dissolution and diffusion of ciprofloxacin. Co-delivery of antibiotics and DNase using a single inhalable particle system may be a promising strategy for local antipseudomonal therapy in the CF airway.

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